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Serum and Testicular Testosterone and Androgen Binding Protein Profiles Following Subchronic Treatment with Carbendazim

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16. ABSTRACT
While the general toxicity of the benzimidazole pesticides for mammals is low, one of these compounds, Carbendazim causes degeneration of testicular tissue and decreases spermatogenic activity at doses well below the LD_{50} value (Barnes et al., 1983). A study conducted by Carter et al. (1987) showed that treatment with 400mg/kg/d MBC resulted in severe seminiferous tubular atrophy and infertility. Since spermatogenesis is an androgen dependent process, we characterized the effects of MBC (0-400mg/kg/d) on the endocrine function of the rat testes. Following sub-chronic (85d) exposure, serum hormones (TSH, LH, FSH and Prl) were measured as were androgen binding protein (ABP) and testosterone in testicular fluids [interstitial fluid (IF) and seminiferous tubule fluid (SNF)]. In addition, the functional capacity of the Leydig cell to secrete testosterone was assessed in vitro following an hCG challenge. Subchronic treatment with MBC at doses of 50-100mg/kg/d had no effect on pituitary or testicular hormone concentrations. 200mg/kg/d elevated the testosterone concentration in the SNF and the ABP concentration in both the IF and SNF without affecting serum testosterone or ABP concentrations. The 400/kg mg dose resulted in increased concentration of both testosterone and ABP in the IF and SNF and elevated serum ABP, with no change in serum testosterone. This endocrine profile is consistent with the testicular atrophy and "Sertoli cell-only" syndrome seen in these animals as reported by Gray et al. (in prep.). We conclude that SNF testosterone may be a result of two factors, 1) increased IF testosterone concentrations and 2) decreased testosterone outflow from the testis to the general circulation. Also, increased ABP in the IF may reflect a change in the relative secretion of ABP into the IF and the seminiferous tubules.

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Serum and Testicular Testosterone and Androgen Binding Protein Profiles following Subchronic Treatment with Carbendazim[1]

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Serum and Testicular Testosterone and Androgen Binding Protein Profiles following Subchronic Treatment with Carbendazim. REHNBERG, G. L., COOPER, R. L., GOLDMAN, J. M., GRAY, L. E., HEIN, J. F., AND MCELROY, W. K. (1989). *Toxicol. Appl. Pharmacol.* **101,** 55–61. While the general toxicity of the benzimidazole pesticides for mammals is low, one of these compounds, carbendazim (MBC), causes degeneration of testicular tissue and decreases spermatogenic activity at doses well below the LD_{50} value. A study conducted by S. D. Carter, R. A. Hess, and J. W. Laskey (1987, *Biol. Reprod.* **37,** 709–717) showed that treatment with 400 mg/kg/day MBC resulted in severe seminiferous tubular atrophy and infertility. Since spermatogenesis is an androgen-dependent process, we characterized the effects of MBC (0–400 mg/kg/day) on the endocrine function of the rat testes. Following subchronic (85 day) exposure, serum hormones (TSH, LH, FSH, and Prl) were measured as were androgen binding protein (ABP) and testosterone in testicular fluids (interstitial fluid and seminiferous tubule fluid). In addition, the functional capacity of the Leydig cell to secrete testosterone was assessed *in vitro* following an hCG challenge. Subchronic treatment with MBC at doses of 50–100 mg/kg/day had no effect on pituitary or testicular hormone concentrations: 200 mg/kg/day elevated the testosterone concentration in the seminiferous tubule fluid and the ABP concentration in both the interstitial fluid and the seminiferous tubule fluid without affecting serum testosterone or ABP concentrations. The 400 mg/kg/day dose resulted in increased concentration of both testosterone and ABP in the interstitial fluid and seminiferous tubule fluid and elevated serum ABP, with no change in serum testosterone. This endocrine profile is consistent with the testicular atrophy and "Sertoli cell-only" syndrome seen in these animals as reported by Gray *et al.* (1987, *Toxicologist* **7,** 717). We conclude that seminiferous tubule fluid testosterone may be a result of two factors: (1) increased interstitial fluid testosterone concentrations and (2) decreased testosterone outflow from the testis to the general circulation. Also, increased ABP in the interstitial fluid may reflect a change in the relative secretion of ABP into the interstitial fluid and the seminiferous tubules. © 1989 Academic Press, Inc.

Carbendazim (MBC), or methyl 2-benzimidazolearbamate, was introduced as a commercial fungicide in 1972. MBC is a metabolite of both benomyl and thiophanate-methyl and is recognized as the chemical entity which is mainly responsible for the fungitoxic activity of the benzimidazole fungicides. The antifungal effects of MBC are described as practically similar to benomyl and thiophanate-methyl. It is a broad spectrum, systemic fungicide which is active against molds, rots, and blight (Vettorazzi, 1976).

The acute toxicity of benzimidazole pesticides in mammals is low (LD_{50}, male rats, per oral is >15 g/kg; Seiler, 1975). These compounds are readily absorbed from the

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gastrointestinal tract; however, absorption does vary. MBC is absorbed more readily from the gastrointestinal tract than its parent compound, benomyl (Styles and Garner, 1974). No single organ seems to accumulate these compounds, and there seem to be no barriers to their penetration (Noguchi *et al.*, 1971). However, these compounds do not accumulate in animal tissue (Gardiner *et al.*, 1974). It has been reported (WHO, 1973) that in subacute studies with MBC, no effects were seen on biochemical and histological parameters. Chronic feeding, acute inhalation, and acute oral toxicity studies indicate that the parent compound (benomyl) and/or its metabolite, MBC, reaches the testes and is capable of causing degeneration of testicular tissue and decreasing spermatogenic activity (Barnes *et al.*, 1983). Parvinen and Kormano (1974), using transillumination techniques, observed premature release of spermatids following MBC treatment. They postulated that there was a uniform disturbance of Sertoli cell function resulting in the breakdown of Sertoli cell spermatid connections, with the spermatids probably released in the order of the strength of their connections.

The Sertoli cell has a key function in the initiation, maintenance, and control of spermatogenesis (Ritzen *et al.*, 1982). Androgen binding protein (ABP), a secretory product of the Sertoli cell, has been used to study the function of the Sertoli cells and the seminiferous tubules (Bardin *et al.*, 1981). ABP is secreted bidirectionally from the Sertoli cells, with 80% secreted into the tubular lumen and 20% released into the blood and lymphatics of the testis. Several studies have indicated that this bidirectional release can be selectively altered by hormones, drugs, and testicular disease. Sertoli cell activity and the production of ABP by the Sertoli cell are closely related to the stage of the spermatogenic cycle (Sharpe and Bartlett, 1987), with greater amounts of ABP secreted during Stages VII–XIII of the cycle (Ritzen *et al.*, 1982).

Spermatogenesis is dependent on testosterone secreted by the Leydig cell. Leydig cell function is dependent upon the molecular components within the interstitial fluid (IF) that surrounds these cells. Thus local changes in the volume or molecular components of this fluid may result in altered Leydig cell function.

Our laboratory has been characterizing the effects of MBC on the hypothalamic–pituitary–gonadal axis using a subchronic dosing regimen that covers puberty through adulthood in male Long–Evans rats. In these studies, the dose-dependent effects of MBC (0–400 mg/kg) are revealed on spermatogenesis (Gray *et al.*, 1987) and hypothalamic–pituitary function (Goldman *et al.*, 1989). Since spermatogenesis is an androgen-dependent process, this report focuses on characterizing the endocrine function of the testes following MBC-induced disruption of spermatogenesis.

In addition to measuring testosterone and ABP in the serum, we determined the concentration of these hormones in the testes by assaying both IF and seminiferous tubule fluid (SNF). To further evaluate Leydig cell function in MBC-treated rats, we determined the ability of the testes to secrete testosterone *in vitro*, with and without hCG stimulation.

MATERIALS AND METHODS

Ninety-day-old pregnant Long–Evans hooded rats were obtained from Charles River Breeding Laboratories on Day 3 of gestation. They were maintained on a 12-hr: 12-hr photoperiod with lights on at 0600 EST. Purina Lab Chow No. 5001 and water were available *ad libitum*. On Day 6 postpartum, litters were culled to eight animals (four males and four females). Pups were weaned on Day 21 postpartum and randomly assigned to treatment groups of equal means and variances in body weight. Animals were subsequently weighed twice weekly for the duration of the study. Dosing, by oral gavage, was started on Day 21 after birth. Dose groups consisted of control, 50, 100, 200, and 400 mg/kg body wt/day. Animals received the appropriate dose in 5 μl/g body wt until they reached a body weight of 200 g, at which time the volume was decreased to 2.5 μl/g for the duration of the study. Dosing was continued daily for 85 consecutive days. Males were housed in unisexual groups (two per cage). Rats were rapidly decapitated between 0900 and 1200 EST. Trunk blood was collected in 16 × 125-mm serum separation tubes (Becton–Dickinson, Rutherford, NJ) and centrifuged (3000 rpm, 4°C for 30 min). Serum was

stored at −60°C until subsequently assayed for the various hormones.

One epididymis was removed, trimmed of fat, weighed, and frozen at −60°C until testosterone and androgen binding protein (ABP) analysis. A testis was also removed, cleaned, and weighed. Testicular IF was collected using a modification of the procedures described by Sharpe and Cooper (1983, 1984) and Turner *et al.* (1984). Briefly, a small incision was made in the caudal end of the testicular capsule taking care not to damage the seminiferous tubules. The testis was then suspended by a thread in a 10-ml syringe barrel so that the caudal end of the testis was slightly above the syringe hub. A preweighed microfuge tube was attached to the hub of the syringe. The suspended testis was then centrifuged at 30*g* for 45 min (4°C). The volume of the collected IF was determined by weighing the microfuge tube again after it was separated from the syringe barrel. Two hundred microliters of phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) was added to the IF. The IF was centrifuged again to remove cellular debris. The fluid was then stored at −60°C until subsequent assays for testosterone and ABP.

Following IF collection, basal and hCG-stimulated testosterone release were determined using this same testis. The testis was decapsulated and placed in a glass scintillation vial containing 2.5 ml medium-199. Each vial was then placed in a shaking water bath (37°C) for 1 hr. After this time an aliquot of the medium was removed and 100 IU of hCG added. Sequential aliquots were then taken at 1, 2, and 3 hr after the addition of hCG. All aliquots were frozen and stored (−60°C) until assayed for testosterone. Following *in vitro* hCG stimulation, SNF was collected from the same testis according to the method described by Turner *et al.* (1984). The testis was removed from the incubation medium, blotted dry, and then washed in medium-199 four times, being blotted dry between each wash. The rinsed and blotted tubules were extruded through a 10-ml syringe into a conical centrifuge tube. The disrupted tubules were then centrifuged at 30,000*g* for 30 min (4°C) and the supernatant overlying the compressed tubules was transferred to a preweighed vial. A 200-μl aliquot of PBS containing 2% BSA was then added. The fluid was stored frozen at −60°C until assayed for testosterone and ABP.

Caput epididymal tissue was processed using a modification of Vogel *et al.* (1983). This tissue was homogenized in 9 vol of TCG buffer (50 mM Tris base, 50 mM $CaCl_2$, and 10% glycerol; v/v, pH 7.4) and centrifuged at 35,000*g* for 45 min (4°C). The supernatants were kept frozen at −60°C until assayed for testosterone and ABP.

Hormone Assays

Testosterone concentrations in serum, IF, and SNF were determined using radioimmunoassay kits obtained from Diagnostic Products Corp. (Los Angeles, CA). The antiserum provided with this kit is specific for testosterone and the cross reactivity with 5α-dihydrotestosterone is less than 8%. Just prior to the assay, IF and SNF were appropriately diluted with PBS (0.1 M) with 2% BSA. Intra- and interassay variances were 4 and 3%, respectively.

ABP concentrations in serum, IF, SNF, and epididymal cytosol were measured with reagents supplied by the National Hormone and Pituitary Program, NICHD (Ref. Prep., WR-R1), according to the procedures described by Gunsalus *et al.* (1978, 1981). Samples were appropriately diluted with 0.01 M PBS/0.15 M NaCl, pH 7.4, containing 0.5% BSA. Rat ABP was iodinated by the iodogen method of Fracker and Speck (1978), and purified on a Sephadex G-75 column using sodium phosphate buffer (0.01 M). Immediately prior to assay, the labeled hormone was fractionated on a DEAE Bio-Gel A column (Bio-Rad Laboratories). Labeled ABP, antiserum, and sample were then incubated for 72 hr at 4°C. Goat anti-rabbit γ-globulin was used as the second antibody (Calbiochem). For this study, all samples were analyzed in the same assay. The intraassay variance was 9.6%, and the minimal detectable dose was 0.59 ng/tube.

Statistics

Significant effects among groups were tested by analysis of variance under the General Linear Models procedure of the Statistical Analysis System (SAS) package, with the alpha level set at 0.05.

RESULTS

Testis weights were decreased 37% in the 200 and 400 mg/kg/day dose groups, while caput epididymal weights in the same groups were also significantly lower than those of controls (Table 1). Interstitial fluid volumes were variable with a significant reduction in volume present only in the highest dose group. The SNF volumes of the 200 and 400 mg/kg/day groups were significantly lower than control rats' SNF volumes.

Although serum concentrations of testosterone did not change with MBC treatment, intratesticular testosterone concentrations were affected (Table 2). Testosterone levels in SNF were elevated in animals exposed to 200 and 400 mg MBC/kg/day. Similarly, IF testosterone concentrations were higher in the 400 mg/kg/day exposure group.

The results of hCG stimulation of the decapsulated testes are shown in Table 3. There

TABLE 1

ORGAN WEIGHTS IN MALE RATS TREATED WITH CARBENDAZIM (MBC)

	Dose (mg/kg/day)				
	Control	50	100	200	400
Testis (g)	1.783 ± 0.058	1.735 ± 0.057	1.648 ± 0.063	1.122 ± 0.105[a]	1.132 ± 0.064[a]
Caput epididymis (g)	0.277 ± 0.005	0.273 ± 0.014	0.273 ± 0.007	0.240 ± 0.015[b]	0.219 ± 0.008[a]
Interstitial fluid (mg)	79.6 ± 7.2	87.0 ± 9.6	91.1 ± 8.5	98.2 ± 15.6	55.9 ± 9.9[c]
Seminiferous tubule fluid (mg)	164.3 ± 13.2	147.1 ± 10.4	146.0 ± 11.4	125.1 ± 11.6[c]	110.8 ± 9.3[b]

Note. Values are means ± SEM of seven or eight rats.
[a] $p < 0.0001$.
[b] $p < 0.01$.
[c] $p < 0.05$.

was no difference among the groups in basal testosterone release. However, hCG stimulation resulted in a significantly greater amount of testosterone synthesis/release in the testes obtained from the animals receiving 200 and 400 mg/kg MBC at each time point measured.

The serum ABP concentration was significantly increased in the 400 mg/kg/day group (Table 4). The concentration of ABP in both the IF and the SNF of the 200 and 400 mg/kg/day groups was significantly greater than that observed in the control animals. In contrast, caput epididymal concentration of ABP was unaffected by MBC treatment.

DISCUSSION

Previous studies have shown that MBC is an exfoliative antispermatogenic agent in that it causes premature sloughing of spermatids (Parvinen and Kormano, 1974). Other exfoliative antispermatogenic agents, such as tolnidamine, have been reported to alter seminiferous tubular function as evidenced by reduced testis weights and altered ABP concentrations in serum, testis, and epididymis. Leydig cell function is also presumed to be unaffected as treatment with these agents does not alter serum testosterone, and only a minimal increase in the concentration of se-

TABLE 2

EFFECT OF CARBENDAZIM (MBC) TREATMENT ON TESTOSTERONE CONCENTRATIONS IN SERUM, INTERSTITIAL FLUID, AND SEMINIFEROUS TUBULE FLUID

	Dose (mg/kg/day)				
	Control	50	100	200	400
Serum (ng/ml)	1.99 ± 0.36	2.22 ± 0.38	2.14 ± 0.62	2.09 ± 0.35	3.19 ± 1.13
Interstitial fluid (ng/ml)	222 ± 28	247 ± 34	228 ± 42	287 ± 59	423 ± 111[a]
Seminiferous tubule fluid (ng/ml)	816 ± 54	875 ± 51	918 ± 78	1004 ± 75[b]	1011 ± 33[b]

Note. Values are means ± SEM of seven or eight rats.
[a] $p < 0.01$.
[b] $p < 0.05$.

TABLE 3

EFFECT OF CARBENDAZIM (MBC) TREATMENT ON hCG-STIMULATED TESTOSTERONE RELEASE FROM DECAPSULATED TESTES

	Dose (mg/kg/day)				
Incubation time	Control	50	100	200	400
Basal (ng/testis)	126 ± 11	142 ± 13	151 ± 34	119 ± 15	185 ± 42
1 hr (ng/testis)	530 ± 43	539 ± 54	619 ± 75	893 ± 131[a]	1056 ± 181[b]
2 hr (ng/testis)	846 ± 57	980 ± 113	1045 ± 86	1698 ± 298[b]	1856 ± 228[c]
3 hr (ng/testis)	1078 ± 81	1261 ± 113	1332 ± 127	2192 ± 377[c]	2565 ± 302[c]

Note. Values are means ± SEM of seven or eight rats.
[a] $p < 0.01$.
[b] $p < 0.001$.
[c] $p < 0.0001$.

rum LH and FSH is observed (Spitz *et al.*, 1985).

In the present study, MBC treatment altered the concentration of testosterone and ABP within the different testicular compartments. The dose of MBC required to produce these changes was 200 mg/kg/day or greater. Exposure to MBC also produced changes in pituitary and serum gonadotropin concentrations, namely serum FSH and pituitary LH, both of which were significantly elevated at a dose of 400 mg/kg/day (Goldman *et al.*, 1989). Although further studies are needed to determine possible short-term acute effects of MBC on the hypothalamic–pituitary control of testicular function, at present the composite changes observed in the animals receiving 400 mg/kg/day indicate that this compound produces changes within the reproductive system indicative of the "Sertoli cell-only" syndrome in which serum LH and testosterone concentrations are within the range of controls and serum FSH concentration is slightly elevated. These findings are consistent with those reported following treatment with other exfoliative agents (Spitz *et al.*, 1985), with the exception that we found no effect of MBC on epididymal ABP concentrations.

TABLE 4

EFFECT OF CARBENDAZIM (MBC) TREATMENT ON ABP CONCENTRATIONS IN SERUM, INTERSTITIAL FLUID, SEMINIFEROUS TUBULE FLUID, AND CAPUT EPIDIDYMAL TISSUE

	Dose (mg/kg/day)				
	Control	50	100	200	400
Serum (ng/ml)	222 ± 10	239 ± 14	216 ± 8	242 ± 7	275 ± 15[a]
Interstitial fluid (ng/ml)	1039 ± 70	940 ± 38	912 ± 44	1382 ± 181[a]	1414 ± 167[a]
Seminiferous tubule fluid (ng/ml)	3143 ± 124	3708 ± 452	3539 ± 567	4132 ± 494[a]	4632 ± 712[b]
Caput epididymis (ng/mg)	30.9 ± 3.4	35.2 ± 3.8	29.3 ± 2.8	34.2 ± 8.1	29.4 ± 6.8

Note. Values are means ± SEM of seven or eight rats.
[a] $p < 0.05$.
[b] $p < 0.01$.

The increased concentration of testosterone in the IF and SNF of the two highest dose groups was insufficient to elevate peripheral serum testosterone, indicating that the outflow of testosterone from the testis of the treated animal was reduced. This may be due to a decrease in blood flow. Reduced blood flow limits the amount of testosterone leaving the testis and it has been reported that reduced blood flow results in an increase in interstitial fluid testosterone (Setchell and Galil, 1983). Setchell and Galil (1983) concluded that a decreased mass of tubules in the testis may deter testis blood flow and the Leydig cell must manage with what the tubules require. Damber *et al.* (1985) also reported a positive correlation between blood flow and the secretion of testosterone from the rat testis.

Basal synthesis/release of testosterone *in vitro* showed a slight but not significant increase; however, hCG-stimulated testosterone showed marked increases in the two highest dose groups. This finding further indicates that Leydig cell function was also altered, either directly or indirectly, by treatment with MBC. The greater release of testosterone by the Leydig cells of the animals receiving 200 and 400 mg/kg/day in response to hCG stimulation *in vitro* was not the result of altered serum LH. Rather, this change in testosterone release may have been induced by damage to the seminiferous epithelium. Normally, the seminiferous tubules exert a controlling influence on Leydig cell function, and this control is altered when the seminiferous epithelium is functionally impaired. Gray *et al.* (1987) observed severely depleted germ cell complements in the two higher dose groups and in many cases, especially in the 400 mg/kg/day group, only Sertoli cells were observed. The present findings were also consistent with those of Aoki and Fawcett (1978), who demonstrated that focal lesions of the seminiferous tubules result in Leydig cell hypertrophy in the area immediately surrounding the damaged tissue. Syed *et al.* (1986) showed that a factor(s) produced by isolated seminiferous tubules from the cryptorchid testis stimulates testosterone production *in vitro* by crude interstitial cells, indicating the disappearance of the inhibitory influence of seminiferous tubules on testosterone production.

In summary, exposure to MBC results in altered testosterone and androgen binding hormone concentrations in the two fluid compartments of the testis. These changes reflect alterations in both Leydig cell and Sertoli cell function that are most likely the consequence of damage to the seminiferous epithelium.

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